

We claim:

5        1. A method for identifying an atom of a common ligand mimic that is proximal to an interface region;

10        wherein the enzyme can bind a common ligand (CL) or a common ligand mimic (CL mimic) at a common ligand site (CL site) and can bind a specificity ligand (SL) at an adjacent specificity ligand site (SL site);

15        wherein an interface region is defined as the atoms of the enzyme between the CL site and SL site, and atoms of an SL if bound to the enzyme;

20        wherein the enzyme can catalyze a reaction mechanism involving the SL and a reactive atom of the CL; and

25        wherein a CL reactive region is defined as the reactive atom of the CL and CL atoms immediately adjacent to the reactive atom or CL atoms immediately adjacent to the SL;

comprising the steps of

30        (a)        identifying an atom of the interface region, comprising the steps of

              (1)        binding a CL to the CL site of the enzyme;

35        (2)        perturbing an atom of the CL reactive region; and

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5 (3) identifying an NMR cross-peak corresponding to an atom that is perturbed by the perturbation of the atom of the CL reactive region, thereby identifying an atom of the interface region; then

10 (b) identifying an atom in the CL mimic that is proximal to the interface region, comprising the steps of

15 (1) binding a CL mimic to the CL site;

(2) perturbing the interface atom identified in step (a); and

20 (3) identifying an NMR cross-peak corresponding to an atom of the CL mimic that is perturbed by the perturbation of the interface atom, thereby identifying an atom of the CL mimic that is proximal to the interface region.

25 2. The method of claim 1, wherein the enzyme has a monomer molecular weight greater than 20 kD.

30 3. The method of claim 2, wherein the enzyme has a monomer molecular weight greater than 35 kD.

4. The method of claim 1, wherein the enzyme has a complete molecular weight greater than 50 kD.

35 5. The method of claim 4, wherein the enzyme has a complete molecular weight greater than 100 kD.

6. The method of claim 1, wherein the enzyme is from a human pathogen.

5 7. The method of claim 1, wherein the enzyme is from bacteria.

8. The method of claim 1, wherein the enzyme is a dehydrogenase.

10 9. The method of claim 1, wherein the enzyme is a kinase.

15 10. The method of claim 1, wherein the atom of the interface region in step (b)(2) is an atom of the enzyme.

20 11. The method of claim 1, wherein the atom of the interface region in step (b)(2) is an atom of an SL bound to the enzyme.

12. The method of claim 1, wherein the CL is a cofactor.

25 13. The method of claim 12, wherein the CL is SAM (S-adenosyl methionine).

14. The method of claim 12, wherein the cofactor contains a nucleotide.

30 15. The method of claim 14, wherein the CL is selected from the group consisting of NAD<sup>+</sup>, NADH, NADP<sup>+</sup>, NADPH, ATP and ADP.

16. The method of claim 12, wherein the CL is selected from the group consisting of farnesyl, geranyl, geranyl-geranyl and ubiquitin.

5 17. The method of claim 1, wherein the atom of the CL reactive region in step (a)(2) is the reactive atom of the CL.

10 18. The method of claim 1, wherein the atom of the reactive region in step (a)(2) is a CL atom immediately adjacent to the reactive atom.

15 19. The method of claim 1, wherein the atom of the reactive region in step (a)(2) is a CL atom immediately adjacent to the SL.

20 20. The method of claim 1, wherein a perturbing step is achieved by chemically altering an atom.

21. The method of claim 20, wherein an atom of the CL reactive region is chemically altered by replacing a hydrogen atom with a deuterium atom.

25 22. The method of claim 20, wherein an atom of the enzyme in the interface region is chemically altered by site-directed mutagenesis.

30 23. The method of claim 1, wherein a perturbing step is achieved by chemically altering an atom immediately adjacent to the perturbed atom.

24. The method of claim 23, wherein the chemical alteration is an introduction of an atom selected from the group consisting of a paramagnetic atom and a quadrupolar atom.

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25. The method of claim 1, wherein a perturbing step is achieved by irradiating an atom with radio frequency energy.

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26. The method of claim 1, wherein a perturbing step results in a nuclear Overhauser enhancement effect.

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27. The method of claim 1, wherein a perturbing step results in an NMR cross-peak intensity or shape change.

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28. The method of claim 1, wherein a perturbing step results in a relaxation effect.

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29. The method of claim 1, wherein a perturbing step results in an NMR cross-peak chemical shift change.

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30. The method of claim 1, wherein an NMR cross-peak is identified using a multidimensional multinuclear method, wherein the transfer of magnetization to protons is only to or from amide protons.

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31. The method of claim 1, wherein an NMR cross-peak is identified using a multidimensional multinuclear method, wherein the detectable atoms are the NH protons of protein at an amino acid selected from the group consisting of Asn, Gln, Arg and His.

32. The method of claim 1, wherein an NMR cross-peak is identified using a multidimensional multinuclear method, wherein the detectable atoms are the methyl protons of protein specifically  $^{13}\text{C}-^1\text{H}$ , labeled at an amino acid selected from the group consisting of Leu, Thr, Ile, Val, Ala and Met.

5 33. The method of claim 1, wherein an NMR cross-peak is identified using a multidimensional multinuclear method that includes a  $^1\text{H}-^{15}\text{N}$  correlation.

10 34. The method of claim 33, wherein the NMR method is a  $^1\text{H}-^{15}\text{N}$  correlation and nuclear Overhauser enhancement spectroscopy experiment.

15 35. The method of claim 1, wherein an NMR cross-peak is identified using a multidimensional multinuclear method that includes a  $^1\text{H}-^{13}\text{C}$  correlation.

20 36. The method of claim 33, wherein the NMR method is an HNCA experiment.

25 37. The method of claim 1, wherein an NMR cross-peak is identified using an NMR method that includes a  $\{^1\text{H},^1\text{H}\}$  NOESY step.

30 38. The method of claim 37, further comprising the step of introducing a third dimension for  $^{15}\text{N}$  or  $^{13}\text{C}$  chemical shift.

35 39. The method of claim 37, wherein diagnostic  $^1\text{H}-^{13}\text{C}$  or  $^1\text{H}-^{15}\text{N}$  one bond coupling constants are obtained by not decoupling to a heteroatom in one of the two dimensions.

40. The method of claim 37, further comprising the step of using 2D  $^{13}\text{C}$ - $^1\text{H}$  or  $^{15}\text{N}$ - $^1\text{H}$  HMQC or HSQC- $\{^1\text{H},^1\text{H}\}$  NOESY.

5 41. The method of claim 1, wherein an NMR cross-peak is identified using an NMR experiment that uses transverse relaxation-optimized spectroscopy (TROSY), whereby narrow line widths are achieved.

10 42. The method of claim 1, wherein an NMR cross-peak is identified using an NMR experiment that uses deuterium labeling and decoupling, whereby narrow line widths are achieved.

15 43. A method for identifying an atom of a common ligand mimic that is proximal to an interface region;

20 wherein the enzyme can bind a common ligand (CL) or a common ligand mimic (CL mimic) at a common ligand site (CL site) and can bind a specificity ligand (SL) at an adjacent specificity ligand site (SL site);

25 wherein an interface region is defined as the atoms of the enzyme between the CL site and SL site, and atoms of an SL if bound to the enzyme;

30 wherein the enzyme can catalyze a reaction mechanism involving the SL and a reactive atom of the CL;

35 wherein a CL reactive region is defined as the reactive atom of the CL and CL atoms

immediately adjacent to the reactive atom or CL atoms immediately adjacent to the SL;

5 and wherein an atom of the interface region has been identified;

comprising the steps of

10 (1) binding a CL mimic to the CL site;

(2) perturbing the identified atom of the interface region; and

15 (3) identifying an NMR cross-peak corresponding to an atom of the CL mimic that is perturbed by the perturbation of the interface atom, thereby identifying an atom of the CL mimic that is proximal to the interface region.

20 44. A method for identifying an atom of a common ligand mimic that is proximal to an interface region;

25 wherein the enzyme can bind a common ligand (CL) or a common ligand mimic (CL mimic) at a common ligand site (CL site) and can bind a specificity ligand (SL) at an adjacent specificity ligand site (SL site);

30 wherein an interface region is defined as the atoms of the enzyme between the CL site and SL site, and atoms of an SL if bound to the enzyme;

wherein the enzyme can catalyze a reaction mechanism involving the SL and a reactive atom of the CL;

5                   wherein a CL reactive region is defined as the reactive atom of the CL and CL atoms immediately adjacent to the reactive atom or CL atoms immediately adjacent to the SL;

10                  comprising the steps of

15                  (1)        binding a CL to the CL site in the presence of unbound CL mimic;

20                  (2)        perturbing an atom of the CL, whereby energy is transferred from the CL atom to the interface region;

25                  (3)        allowing the CL to unbind and a CL mimic to bind at the same CL site, whereby energy is transferred from the interface region to perturb an atom in the CL mimic; and

                     (4)        identifying an NMR cross-peak corresponding to the atom of the CL mimic perturbed in step (3), thereby identifying an atom of the CL mimic that is proximal to the interface region.

45. A method for identifying an atom of a specificity ligand mimic that is proximal to an interface region;

5                   wherein the enzyme can bind a specificity ligand (SL) or a specificity ligand mimic (SL mimic) at a specificity ligand site (SL site) and can bind a common ligand (CL) or common ligand mimic (CLM) at an adjacent common ligand site (CL site);

10                   wherein an interface region is defined as the atoms of the enzyme between the SL site and CL site, and atoms of a CL if bound to the enzyme;

15                   wherein the enzyme can catalyze a reaction mechanism involving a CL and a reactive atom of a SL; and

20                   wherein a SL reactive region is defined as the reactive atom of the SL and SL atoms immediately adjacent to the reactive atom or SL atoms immediately adjacent to the CL;

25                   comprising the steps of

(a)                identifying an atom of the interface region, comprising the steps of

30                   (1) binding an SL to the SL site of the enzyme;

                     (2) perturbing an atom of the SL reactive region; and

5 (3) identifying an NMR cross-peak corresponding to an atom that is perturbed by the perturbation of the atom of the SL reactive region, thereby identifying an atom of the interface region; then

10 (b) identifying an atom in the SL mimic that is proximal to the interface region, comprising the steps of

15 (1) binding an SL mimic to the SL site;

20 (2) perturbing the interface atom identified in step (a); and

25 (3) identifying an NMR cross-peak corresponding to an atom of the SL mimic that is perturbed by the perturbation of the interface atom, thereby identifying an atom of the SL mimic that is proximal to the interface region.

46. A method for identifying an atom of a first ligand mimic that is proximal to an interface region;

30 wherein the enzyme can bind a first ligand (L1) or a first ligand mimic (L1 mimic) at a first ligand site (L1 site) and can bind a second ligand (L2) at an adjacent second ligand site (L2 site);

35 wherein an interface region is defined as the atoms of the enzyme between the L1 site and L2 site, and atoms of L2 if bound to the enzyme;

wherein the enzyme can catalyze a reaction mechanism involving the L2 and L1; and

wherein a L1 reactive region is defined as the reactive atom of L1, and L1 atoms immediately adjacent to the reactive atom or L1 atoms immediately adjacent to L2;

comprising the steps of

(a) identifying an atom of the interface region, comprising the steps of

(1) binding an L1 to the L1 site of the enzyme;

(2) perturbing an atom of the L1 reactive region; and

(3) identifying an NMR cross-peak corresponding to an atom that is perturbed by the perturbation of the atom of the L1 reactive region, thereby identifying an atom of the interface region; then

(b) identifying an atom in the L1 mimic that is proximal to the interface region, comprising the steps of

(1) binding a L1 mimic to the L1 site;

(2) perturbing the interface atom identified in step (a); and

(3) identifying an NMR cross-peak corresponding to an atom of the L1 mimic that is perturbed by the perturbation of the interface atom, thereby identifying an atom of the L1 mimic that is proximal to the interface region.

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47. A method for generating a focused combinatorial library of bi-ligand compounds that can simultaneously bind to a CL site and an SL site of an enzyme, comprising the steps of

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- (a) performing the method of claim 1 to identify a CL mimic atom that is proximal to the interface region; and
- (b) synthesizing at least two compounds by modifying at least one proximal atom of the CL mimic by attaching a substituent group to the proximal atom.

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48. The method of claim 47, wherein the substituent group contains a linker arm.

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49. The method of claim 48, wherein the linker connects the CL mimic to a second moiety, whereby the CL mimic binds to the CL site and the second moiety binds to the SJ site.

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50. A combinatorial library of bi-ligand compounds obtained by the method of 49.

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52. A method for screening bi-ligand compounds, comprising the steps of

- (a) performing the method of claim 47 to generate a combinatorial library of bi-ligand compounds;
- (b) measuring the binding of the compounds to the enzyme; and
- (c) identifying a compound with greater binding than the CL mimic.

53. A bi-ligand compound identified by the screening method of claim 52.

54. The bi-ligand compound of claim 53, wherein the compound reduces the activity of the enzyme.

55. The bi-ligand compound of claim 53, wherein the compound's binding affinity to the enzyme is at least 200 times greater than the CL mimic's binding affinity.

56. The bi-ligand compound of claim 55, wherein the compound's binding affinity to the enzyme is at least 1000 times greater than the CL mimic's binding affinity.

57. The bi-ligand compound of claim 56, wherein the compound's binding affinity to the enzyme is at least 5000 times greater than the CL mimic's binding affinity.

58. The bi-ligand compound of claim 53,  
wherein the compound's binding affinity is at least 200  
times greater to the enzyme than to another enzyme in the  
same gene family.